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Emotional stress induced by parachute jumping enhances blood nerve growth factor levels and the distribution of nerve growth factor receptors in lymphocytes

(stress/anxiety/hormones)

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ABSTRACT **We examined the plasma nerve growth factor (NGF) level and the distribution of NGF receptors in peripheral lymphocytes of young soldiers (mean age, 20–24 yr) experiencing the thrill of a novice about to make their first parachute jumps. Blood was collected from soldiers who knew they were selected to jump (n = 26), as well as from soldiers who knew they were not selected (n = 17, controls). The former group was sampled the evening before the jump and 20 min after landing. Compared with controls, NGF levels increased 84% in prejump and 107% in postjump sampling. Our studies also showed that the increase of NGF levels preceded the increase of plasma cortisol and adrenocorticotropic hormone. No changes in the baseline levels of circulating interleukin 1β or tumor necrosis factor were found, suggesting that the increased levels of NGF were not correlated with change in these cytokines. Moreover, immunofluorescence analysis demonstrated that parachuting stress enhances the distribution of low-affinity $p75^{LNGFR}$ and high-affinity $p140^{trk}$A NGF receptors in circulating peripheral blood mononuclear cells. These observations suggest that the release of NGF might be involved in the activation of cells of the immune system and is most probably associated with homeostatic adaptive mechanisms, as previously shown for stressed rodents.**

There is accumulating evidence from psychoneuroimmunological studies in both animals and humans that stress results in the concomitant activation of cells from the nervous, endocrine, and immune systems and in the release of diverse biologically active compounds, including glucocorticoids, catecholamines, and neuropeptides (1–4). Results reported from our laboratory indicate that stressful events induce an increase of the specific growth factor nerve growth factor (NGF) into the bloodstream (5) and in defined hypothalamic subareas (5–9) and that the amount of circulating NGF is directly correlated with the number of fighting episodes. Using this model of stress, we have also shown that mast cells (7) and both cortical and chromaffin adrenal gland cells (8) are receptive to the action of endogenously released NGF. In a subsequent study, we reported that the release of NGF into the bloodstream was not simply associated with the expression of aggressive behavior because sera of mice repeatedly experiencing defeat and submission contained higher NGF levels than those from dominant attacking animals (9). This latter observation suggests that stimuli of a psychological nature, most likely associated with anxiety, also trigger the synthesis and/or release of NGF. Whether stress affects baseline NGF level in mice only or in other species as well is not known. Moreover, there is as yet no evidence indicating that stress causes the release of NGF in the human bloodstream.

Parachute jumping is a well-characterized stress model to study emotional and physical stress in humans (10, 11). In the present study, we used this model to determine whether stress in humans affects plasma NGF levels and NGF-receptor (NGF-r) distribution in peripheral blood cells. To assess whether any change of NGF levels was associated with biological mediators involved in NGF synthesis and/or release (12, 13) plasma levels of cortisol, adrenocorticotropic hormone (ACTH), interleukin 1β (IL-1β), and tumor necrosis factor α (TNF-α) were also evaluated. The results showed that the levels of NGF and the expression of NGF-r in young soldiers who experienced their first jump from an aircraft are enhanced both pre- and postjumping and that the increase of NGF precedes the increase of cortisol and ACTH plasma levels. The functional significance of the enhanced NGF levels during emotional and physical stress in humans is discussed.

MATERIALS AND METHODS

Subjects and Procedure. We studied the levels of circulating NGF, cortisol, ACTH, and markers of peripheral blood cells in 43 young (average age 22–24 yr) male soldiers (who had given their informed consent) randomly selected at the military headquarters of Pisa, Italy. Twenty-milliliter blood samples were collected from soldiers who knew they were selected to parachute for their first time (n = 26), as well as from subjects who knew they were not selected (n = 17, controls). The former group was sampled the evening before the jump and 20 min after landing. Blood from control and prejump soldiers was collected between 4 p.m. and 5 p.m. and from postjump soldiers at 10 a.m. Peripheral blood samples were diluted with sterile saline, and peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Lymphoprep gradient at 400 × g for 20 min. PBMC were collected at the interface and washed three times with sterile phosphate-buffered saline (PBS) and used for indirect immunofluorescence, immunoperoxidase, or cell cytometry scan analysis (Becton Dickinson).

Immunoenzymatic and RIAs. NGF levels were measured using a two-site immunoenzymatic assay (ELISA) as described (14). Briefly, Polystyrene 96-well microtiter immunoplates (Nunc) were coated with affinity-purified polyclonal goat anti-NGF antibody. Parallel wells were coated with preimmune goat IgG for evaluation of the nonspecific signal. After 5 hr at 20°C the plates were washed (as in subsequent

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Abbreviations: NGF, nerve growth factor; NGF-r, nerve growth factor receptor; ACTH, adrenocorticotropic hormone; PBMC, peripheral blood mononuclear cells; IL-1β, interleukin 1β.

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steps) with PBS/0.05% Tween 20 and then incubated for 2 hr at 20°C with PBS/0.05% Tween 20/1% fetal calf serum to block nonspecific binding sites. After washing, the NGF standard solutions, ranging from 0 to 1 ng·ml^{-1}, were distributed in each plate. The samples were ultrasonicated at 4°C in the sample buffer and centrifuged at 9500 × *g* for 30 min at 4°C. One hundred microliters of supernatant was added to each well, and the plates were incubated overnight at 4°C. The plates were then washed and 100 μl of monoclonal antibody against NGF, diluted 1:100 in PBS, was added to each well.

After incubation for 4 hr at 20°C, the plates were washed and incubated with biotinylated rat immunoglobulins (1:8000, Zymed). The subsequent incubation with peroxidase-conjugated streptavidin (Zymed) and addition of *o*-phenyldiamine resulted in a colorimetric reaction whose optical density was measured at 490 nm using a Dynatech MR 5000 microplate reader. Specificity for NGF was also assessed by using a recombinant human NGF (Genentech). Recombinant brain-derived neurotrophic factor is not recognized in the ELISA at concentrations up to 20 ng/ml. The biological activity of NGF was evaluated using sympathetic neurons from fetal rat superior cervical ganglia, which are receptive to NGF (12) but not to other neurotrophins (15, 16). The specific NGF neurite outgrowth-promoting effect of human plasma was assessed by adding affinity-purified NGF antibodies to the tissue culture.

Plasma cortisol and ACTH were purchased from GL Chemical (Milan, Italy), and IL-1β and TNF-α plasma were from Genzyme. RIA determinations were done according to the manufacturers' instructions.

Immunocytochemistry and Cell Cytometry Analysis. A mouse anti-human low-affinity (p75^{LNGFR}) monoclonal antibody ME20.4 (17) (IgG clone 200 3 G64) was isolated from cell culture supernatant of hybridoma cell lines (American Type Culture Collection, Rockville, MD) and purified by using a Sepharose CL-4B column coupled to protein A (Pharmacia). Antibodies against NGF- and lymphocyte-specific surface receptor CD19 were purchased from Becton Dickinson, Italy. Phenotypic analyses were done by indirect immunofluorescence and immunoperoxidase using a described procedure (5, 7, 18). Some samples of PBMC were also used for immunoperoxidase localization of p140^{trk}A antibody, provided by William Mobley from University of California at San Francisco. Low-affinity and p140^{trk}A NGF-r-positive cells were counted using 400 cells per sample.

For cytofluorimetric analysis, PBMC were incubated with antibodies to p75^{LNGFR} or CD19 or with purified mouse IgG (controls) for 45 min at 4°C and after several washings in PBS, cells were exposed for 30 min at 4°C to fluorescein-conjugated goat anti-mouse IgG antibody (Sigma). p75^{LNGFR}-positive cells were quantified by an EPICS 541 flow cytometer (Coulter), exciting the 488-nm line of an Argon-ion laser Innova 90 (Coherent Radiation, Palo Alto, CA) with an emitting power of 200 mV and a passing filter of 590 nm. To assess staining specificity, PBMC were incubated with purified anti-mouse IgG followed by exposure to fluorescein-conjugated antibody.

Statistical Analysis. All data were analyzed using nonparametric tests: Mann–Whitney *U* test for unpaired groups and Wilcoxon test for paired groups.

RESULTS

Compared with controls, the levels of NGF in the bloodstream of parachuting soldiers increased 84% (56.6 versus 96.8 pg/ml, $P \leq 0.01$, Mann–Whitney *U* test) in prejump and 107% (56.6 versus 108 pg/ml, $P \leq 0.01$, Mann–Whitney *U* test) in postjump plasma samples (Fig. 1). The NGF levels in unselected soldiers ranged from 36 to 72 pg/ml of serum,

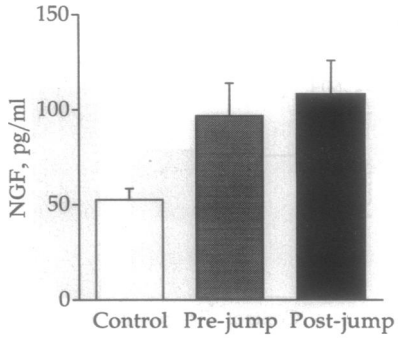

FIG. 1. Levels of NGF in the blood plasma of young soldiers experiencing their first parachute jump from an aircraft. Blood samples were collected from soldiers (n = 26) who knew they were selected to jump the evening before (prejump) and 20 min after landing (postjump). Soldiers who knew they were not selected to jump were used as controls. Plasma samples were tested in triplicate, and the data represent the mean ± SEM. Difference between the mean values is significant ($P \leq 0.01$) according to the Mann–Whitney *U* test.

except for one soldier, who a day before had an allergy attack, and his level of NGF was 93 pg/ml (data not shown). The NGF was biologically active because the addition of plasma from postjump samples to cultured isolated superior cervical ganglia neurons induced neurite outgrowth (results not shown).

To determine whether the increase of NGF was associated with circulating amounts of IL-1β and TNF-α, the levels of these cytokines were measured in the plasma of control subjects as well as in the prejump and postjump samples of the parachutists. No significant changes in the blood levels of IL-1β (20 pg/ml, control versus 17 pg/ml, pre- and postjump) and TNF-α (8 pg/ml, control versus 7 pg/ml, pre- and postjump) were found in the plasma of the parachuting subjects. To evaluate the role of endocrine products on the circulating NGF plasma levels, we measured plasma cortisol and ACTH levels. Results showed that cortisol (Fig. 2) and ACTH (Fig. 3) remained stable in prejumping soldier plasmas but increased after the jump, suggesting that the increases in circulating NGF concentration are not linked to the release of cortisol and ACTH. Thus, the levels of cortisol and ACTH in prejump parachutists (blood collected at 4:00 p.m.) did not change significantly when compared with the levels of cortisol and ACTH in control subjects (blood collected at 4:00 p.m.), whereas the levels of NGF increased in prejump parachutists (blood also collected at 4:00 p.m.), indicating that NGF release preceded the increase of these stress hormones.

To gain more information about the functional role of endogenously released NGF, we studied the distribution of p75^{LNGFR} and lymphocyte-selective markers on PBMC of

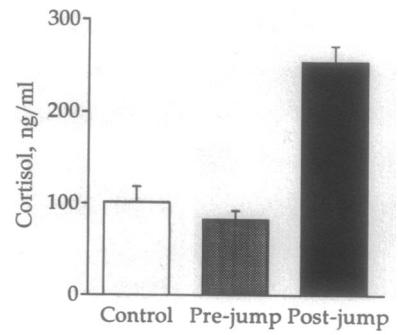

FIG. 2. Levels of cortisol in sera of prejump and postjump parachutists as compared with controls. A significant increase of cortisol was seen in postjump ($P \leq 0.0001$, Mann–Whitney *U* test) but was not seen in prejump parachutists.

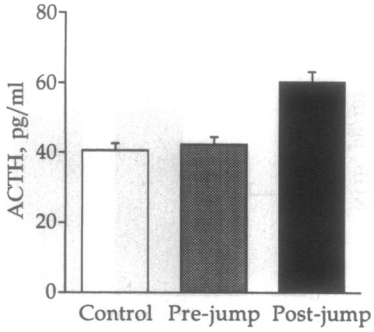

FIG. 3. Levels of ACTH in serum of prejump and postjump parachutists and controls. As for cortisol, the levels of ACTH increase in postjump ($P \leq 0.05$, Mann–Whitney U test) but do not increase in prejump parachutists.

control subjects and experimental subjects. $p75^{LNGFR}$ increased in PBMC of pre- and postjump samples (Fig. 4 *A–D*) with the most intense staining observed in the lymphocytes of postjump parachuters. Quantitative analysis obtained with fluorescence-activated cell sorter cytofluorometry showed a significant increase in the $p75^{LNGFR}$ expressed in PBMC of postjump parachutists (Fig. 5). Immunoperoxidase staining of PBMC from postjump soldiers showed an increased $p140^{trk}A$ immunoreactivity (Fig. 6 *A* and *B*), suggesting a functional link between NGF released and circulating lymphocytes. Moreover, using the two-color fluorescence methodology, NGF-r was found to be colocalized with CD19, suggesting that most cells that express $p75^{LNGFR}$ represent a specific subset of peripheral lymphocytes.

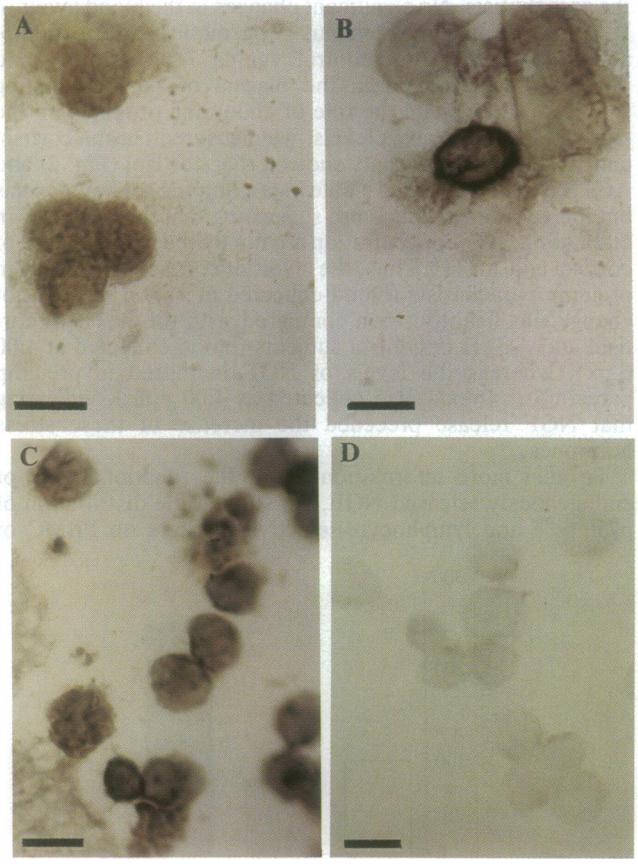

FIG. 4. Immunohistochemical localization of $p75^{LNGFR}$ in PBMC of control subjects (*A*), prejump parachutists (*B*), and postjump parachutists (*C*). (*D*) PBMC nonexposed to $p75^{LNGFR}$ antibodies (negative controls). Note that the immunoreactivity is more intense in cells of postjump parachutists. (Bars = 10 μm.)

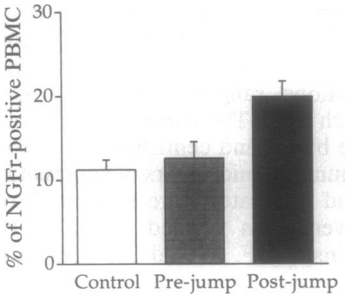

FIG. 5. Cytofluorometric analysis of $p75^{LNGFR}$ expression in PBMC of prejump and postjump parachutists and control subjects, showing a significant increase ($P \leq 0.01$, Mann–Whitney U test) in number of positive PBMC in postjump parachutists as compared with control subjects.

DISCUSSION

Our results showed that the plasma concentration of NGF in soldiers who experienced parachute jumping for the first time increased >100% as compared with the baseline levels of soldiers who were not selected for jumping. The concentration of circulating NGF in parachutists was significantly higher in the evening before the jump, suggesting that the anxiety status triggered the release of circulating NGF. This observation is consistent with our previous observations showing that the plasma NGF levels in fighting mice was higher in subordinate than in dominant mice (9). It was also found that the increase in NGF occurred without parallel increase in cortisol and ACTH, indicating that NGF release is not correlated with these endocrine signals (1–3). Moreover, NGF is not associated with IL-1β or TNF-α (18–20) because the levels of these two cytokines did not vary with this stressful event. Because stressful conditions reportedly

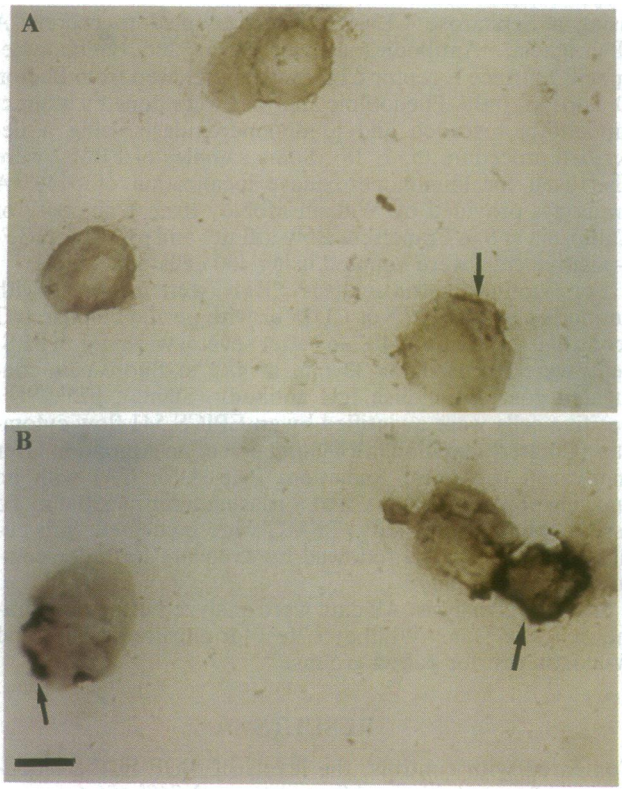

FIG. 6. Immunohistochemical localization of $p140^{trk}A$ in PBMC of control (*A*) and postjump (*B*) soldiers, showing increased immunoreactivity (arrows) in PBMC of postjump parachutists. (Bar = 5 μm.)

act on cells of the immune system (3, 21, 22) and lymphocytes have been shown to respond to the action of NGF (23–27), it was of interest to identify NGF-receptive cells in circulating blood cells. To address this question, we studied the distribution of $p75^{LNGFR}$ in PBMC of controls and parachutists. Qualitative (stain intensity) and quantitative (cell cytometry analysis) immunocytochemistry showed an increase of NGF-r immunoreactivity in PBMC of both prejump and postjump samples.

Although the $p75^{LNGFR}$ is common to other neurotrophins of the NGF family, the expression of this receptor in cells of the nervous, endocrine, and immune systems (27–31) is considered a fairly good marker for identifying NGF-receptive cells. These observations indicate that PBMC are receptive to the endogenously released NGF and suggest a functional link between endogenously released NGF and cells of the immune system. This hypothesis is further supported by immunohistochemical data showing the expression of biologically functional $p140^{trk}A$ in PBMC, which is enhanced in the pre- and postjump results. Whether other neurotrophins of the NGF family (15, 16) are released in the bloodstream of parachutists and whether cells of the immune system are also receptive to these molecules is not known. Evidence reported recently indicated that cells of the immune system, including mast cells, are not receptive to these neurotrophins (32, 33).

A key issue raised by the present observations is the identification of cells releasing NGF. On the basis of our studies (34, §) and those of other investigators,¶ it is conceivable that mast cells, basophils, and lymphocytes, either singly or cooperatively, contribute to the enhanced plasma NGF levels found in parachutists.

It has been reported that NGF enhances the expression of interleukin 2 receptors on human natural killer cells (35), a lymphocyte subtype that plays a well-defined role in the immune surveillance against infection and in stress syndromes (36, 37). Because parachute jumping induces a rapid increase of natural killer cells (17) and these cells appear to be fast-responsive cells to interleukin 2 (18, 30), the endogenous NGF released in the plasma of parachutists might be involved in the activation of natural killer cells. Numerous studies reported in recent years indicate that stressful events, such as situations provoking anxiety (7), mental stress (38), enriched environmental conditions (39), and parturition (40), cause an increase of specific growth factors. Our previous studies in rodents (9) and the present observations in humans clearly indicate that emotional stress enhances plasma NGF levels. These findings are consistent with our recent observation that haloperidol, a major antipsychotic drug acting through the blockade of dopamine receptors and causing a generalized suppression of behavior activity (41), induces a decrease of NGF in both the hypothalamus and the bloodstream (A.L., E.A., D. Della Seta, and F. Cirulli, unpublished work). Moreover, the observation that the enhanced NGF levels precede the increases in cortisol and ACTH supports the hypothesis that NGF might be involved in some early alert mechanism associated with homeostatic adaptation (R. Levi-Montalcini, personal communication). To elucidate the possible functional link between anxiogenic status and circulating NGF levels, the effect of anxyolitic substances, such as galanine (42), neuropeptide Y (43), corticotropin-releasing hormone (CRH) (44–46), and/or anti-stress molecules, such as CRH antagonist (47) should be studied. It would also be of interest to explore the effects of NGF antibodies on physiological changes associated with anxiety—such as heart rate, endocrine variables, or immune alterations.

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§Micera, A., Bracci-Laudiero, L., De Simone, R. & Aloe, L., Third International Conference on Nerve Growth Factor and Related Molecules, April 26–May 1, 1994, Chateau Lake Louise, Lake Louise, AB, Canada.

¶Bracci-Laudiero, L., Cozzolino, F., Torcia, G., Rasi, G. & Aloe, L., Second International Congress of the International Society for Neuroimmunomodulation, May 12–17, 1993, Paestum, Italy.

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